U.S. Fish & Wildlife Service

California Nevada Fish Health Center FY2009 Technical Report:

***Ceratomyxa shasta* myxospore survey of Fall-run Chinook salmon carcasses and sentinel trout trials in Bogus Creek:** Component of joint OSU-Yurok Fisheries-CDFG pilot project testing the effect of carcass removal on *C.shasta* myxospore levels in Bogus Creek, 2008.

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Summary:
A pilot study examining the effect of adult carcass removal on *Ceratomyxa shasta* myxospore release was conducted in Bogus Creek during the fall of 2008. Fall-run Chinook salmon carcasses were removed from the lower reach of Bogus Creek while water samples were assayed for *C.shasta* DNA (actinospore and myxospore input). Study cooperators included Oregon State University, Yurok Tribal Fisheries, California Department of Fish and Game, and the California Nevada Fish Health Center (FHC). The FHC conducted sentinel rainbow trout exposures to determine the presence of infectious actinospore stages and surveyed adult Fall-run Chinook carcasses in Bogus creek for *C.shasta* myxospores. No sentinel fish, which were exposed between 24 Sepember and 05November 2008, became infected with *C.shasta* (no ceratomyxosis and QPCR negative). This data indicates that the actionspore stage was not present in the creek during this period. The incidence of *C.shasta* myxospore detection in intestinal scrapings by phase microscopy was 30% (30 of 100 samples collected between 24October – 14November). *C.shasta* DNA was detected in myxospore-negative (wet mount) samples indicating that either spore concentrations were below the presumed 1400 / gram of scraping detection level or pre-sporogonic stages were present. The number of myxospores per scraping sample varied between 3000 and 14.7 million with no trend for collection date or fish sex. Decomposed carcasses had higher myxospore loads than fresh carcasses. We estimate that a minimum of 4.6 billion myxospores were produced by Fall-run Chinook carcasses present in the Klamath R. basin between Iron Gate Dam and the confluence with the Scott River in December 2008.

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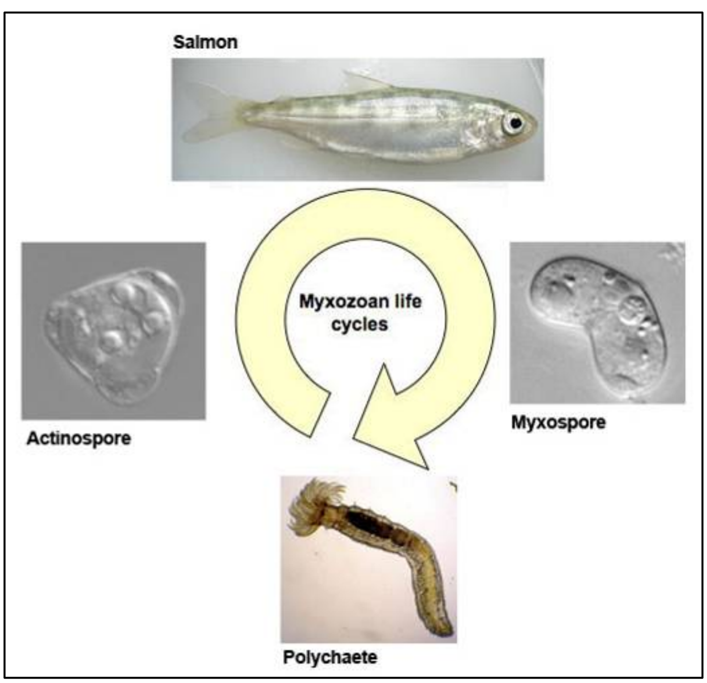
Introduction:
Severe infection, of juvenile Klamath River Chinook salmon and coho salmon, by the myxozoan parasite *Ceratomyxa shasta* may be a contributing factor to declining adult returns in the basin. The incidence of *C. shasta* infection, observed in histological sections of juvenile Chinook collected in the Klamath River above the confluence with the Trinity River between May and July, has ranged from 21 – 35% (Nichols et al. 2008). This incidence is 10 – 27% higher in samples assayed by the more sensitive quantitative polymerase chain reaction assay (QPCR). Approximately 70% of the positive histology samples demonstrated pathology due to the infection. The high prevalence and severity of infection, in native fish that should have high resistance to an endemic disease, indicates this parasite is a key factor limiting salmon recovery in Klamath River.

Ceratomyxa shasta has a complex life cycle, involving an invertebrate (polychaete worm) host as well as salmon (Bartholomew et al. 1997, see diagram below). A section of the lower Klamath River has been identified to be highly infectious to salmon (Stocking et al. 2006) and should be a focus for management actions to disrupt the parasite's life cycle (Fig. 1). In August 2007, a multidisciplinary panel of fish disease experts and fishery managers met to develop a research plan focused on management actions to reduce disease levels (ceratomyxosis) in natural juvenile salmon of the Klamath R. One of the proposed management actions was removal of adult salmon carcasses with the goal of reducing numbers of myxospores released back into the system. The hypothesized effect of this action would be reduced polychaete infection and thus reduction of actinospores later released to infect juvenile fish the following spring.

Figure 1. Lifecycle of *Ceratomyxa shasta* (J. Bartholomew).



The FHC's role in the 2008 carcass removal project was to:

1. Determine if infectious *C.shasta* actinospores, as well as myxospores, are present in lower Bogus creek with sentinel trout challenges (fish are only susceptible to actinospores). This effort is complementary to the water filtration / *Cshasta* DNA assay to track myxospore concentration in the creek by OSU.
2. Survey salmon caracasses in Bogus creek (reach 2) to determine their contribution of myxospores and any correlation with fish sex, state of decomposition or time during the spawning period.

Methods:

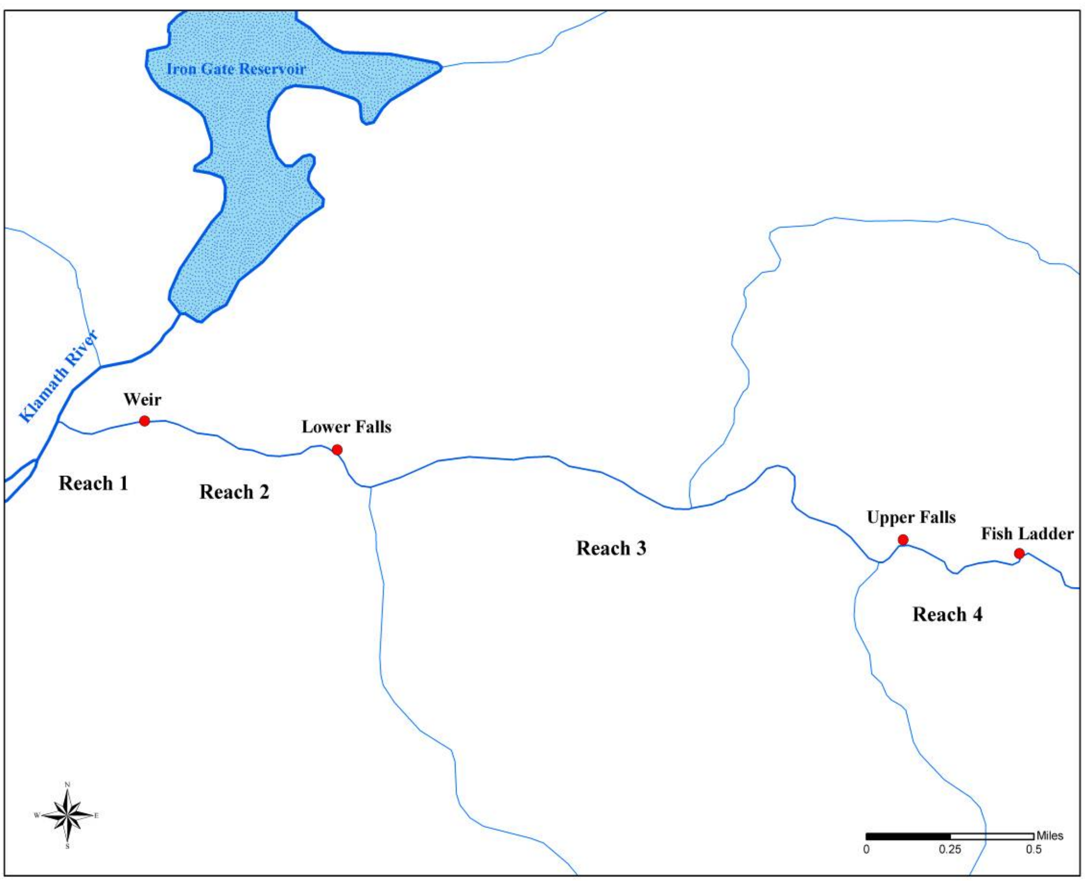
***Sentinels*:** One hundred and sixty-one Rainbow trout (*Oncorhynchus mykiss* ,Roaring River stock, $\bar{x}$ FL = 115.4 mm, $\bar{x}$ Wt = 19.4 g) were received 18Sept2008 from Dr. Jerri Bartholomew (OSU) and transported to the FHC wet laboratory. Fish were held in 800L circular tanks supplied with 7.6L/min of ozonated water ($\bar{x}$ temp. 13.7°C) and aeration. Water temperatures were decreased over time, reflecting the temperature drop in Bogus Creek (Fig.2). Fish were fed a commercial diet (Silvercup Salmon/Steelhead, size 2.5mm) at 1% bodyweight per day. Mean fork lengths and weights were recalculated at each exposure period in order to adjust feeding regimes (Table 1).

Three exposures were conducted below California Department of Fish & Game Klamath River Project Bogus Creek barrier weir (located approximately 0.3 miles upstream from the confluence with the Klamath River) between24Sept and 05Nov (Table 2). This period coincided with the presence of adult Fall-run Chinook salmon (*Oncorhynchus tshawytscha*) returning to spawn in Bogus Creek. Exposures lasted for thirteen days and consisted of 45 fish housed in a 302L aluminum live box. The live box was checked and fish fed once per week by Iron Gate Hatchery personnel. At the end of each exposure period, fish were transported back to the FHC wet laboratory for observation and sampling.

Following the 24Sept exposure, fish were placed directly into a 750L observation tank at temperatures similar to that of Bogus Creek ($\bar{x}$ creek= 13.2°C, $\bar{x}$ holding tank = 15.0°C). Fish from exposures 2 & 3 were given 24h of acclimation at 11.0°C to reduce temperature shock before movement into the 15°C observation tank (Table 3). Fish from all three exposures were held for a 20d observation period (total of 33 days post exposure (dpe)) prior to sampling. Five unexposed (control) fish were sampled at the start of each exposure period.

Carcass myxospore - Chinook carcasses were sampled in reach 2 of Bogus creek (0.6mi reach from weir to waterfall) on 24Oct, 31Oct, 05Nov, and 14Nov (Fig. 1). Fork length, sex, carcass condition (fresh = eyes normal and gill with red or pink color or decomposed), spawn status of female (spawn = few eggs remaining in peritoneal cavity or not spawned) was recorded for each sample. The intestinal tract (junction of stomach and small intestine to rectum) was dissected, placed into individually numbered plastic bags for refrigerated transport back to the laboratory, and held in a 6°C water bath for 2 – 4d prior to processing. On 24Oct, a sample of liver, kidney, and spleen was also collected from adult and placed into a separate pooled-organ bag. Each sample was weighed prior to cutting the intestine into 8 – 12 cm pieces. An intestinal tract content sample (scraping) was obtained by grasping the end of the section with forceps and pushing the backside of a #21 scalpel blade, held at 45° angle, along the outside of the intestine. This process was repeated several times until only the serosa to stratum compactum layers remained. The scraping subsample was weighed, diluted 3x with PBS (4x final dilution), poured into tubes, vortex mixed, and allowed to settle for 1 -3 min. The supernatant in duplicate 10 µL samples was examined for the presence of *C.shasta* myxospores by 40x phase microscopy. Four hemocytometer counts on positive samples quantified the myxospore concentration per gram of sample. Two values are expressed in this report: myxospore / g scraping = derived from the hemocytometer count [(10 x dil.)/ 4 WBC squares X average spore count} and myxospore / scraping = myxospore / g scraping x grams of scraping subsample. We consider the myxospore / scraping to represent the minimum spore load for a given fish. The liver-kidney-spleen samples from myxospore positive carcasses were processed as above except the final dilution was 2x. A subsample of myxospore positive scrapings were assayed by QPCR.

Figure 1. Map of spawning ground survey reaches on Bogus Creek used since the 2003 field season. The weir denotes the location of the Bogus Creek Fish Counting Facility (M. Hampton, CDFG).



Laboratory Assays - At 33 days post-exposure (dpe), all surviving exposed trout were euthanized by an overdose of MS222, measured for fork length and weight, examined for anemia (pale gills) and internal signs of ceratomyxosis such as intestinal hemorrhaging or ascites fluid. The intestinal track and kidney was removed and sampled for PCR assay and histology. QPCR samples were collected from all fish including mortalities. Approximately 5mm of posterior intestine and posterior kidney was removed from each fish with DNA-free tools (washed in DNA-AWAY^{tm} {Molecular BioProducts, San Diego, CA} and rinsed twice in distilled water), combined into individually labeled 2 mL cluster tubes, and stored at -20ºC until DNA extraction. Combined intestine and kidney tissues were digested in 1ml NucPrep Digest Buffer containing 1.25 mg/ml proteinase K (Applied Biosystems, Foster City, CA) at 55ºC for 2 hours with constant shaking. A subsample of digested tissue homogenate diluted 1:33 in molecular grade water and extracted in a 96 well filter plate system (Applied Biosystems Model 6100 Nucleic Acid Prep Station). Extracted DNA was stored at -20ºC. The combined tissues tested for *C. shasta* 18S rDNA using TaqMan Fam-Tamra probe and primers in an Applied Biosystems 7300 Sequence Detection System (Hallett and Bartholomew 2006). Reaction volumes of 30uL, containing 5uL DNA template, were used under the following conditions: 50ºC for 2 min; 95ºC for 10 min; 40 cycles of 95ºC for 15 s and 60ºC for 1 min. Standards, extraction control and no template control wells (MGW) were included on each assay plate (True et al. 2008; Nichols and True 2007). Fluorescent Unit change (Δ Rn) was measured at each cycle and positive test results are reported as Cycle Threshold (Ct), the point when fluorescent signal surpasses normalized background levels. Detection thresholds are set at Δ Rn of 1000 fluorescent units (FU) which occur at (Ct) values of 38-39.

Histological samples were taken from all 33 dpe sentinel fish and fresh mortalities (gills still pink). Both kidney and intestine were removed (after PCR sampling), placed into cassettes labeled with unique identifiers, fixed for 24 h in Davidson's fixative and then transferred to 70% ethanol for storage. Selected specimens were processed for 5µm paraffin sections, stained with hematoxylin and eosin, and examined using bright field microscopy at 40x and 400x magnification.

Results and Discussion:

Sentinel - Trout weight varied between a mean of 19.5 to 21.4 g (Table 1) with $\geq$ 98 % of sentinel fish surviving the 13d exposure (Table 2). Water temperature declined over the study from a daily mean of 13.2° to 8.6°C (Table 3 and Fig. 2). Post-exposure mortality occurred between 19 and 32 dpe in a low percentage of each exposure group (exposure 1 = 8 / 45 (18%), exposure 2 = 4/ 45 (9%), and exposure 3 = 1 / 45 (2%)). The majority of mortalities had skin lesions likely incurred during exposure. *C.shasta* was not detected by QPCR in either non-exposed controls, sentinel survivors or mortalities from all three exposures. Similarly, *C. shasta* was not observed in intestinal tract histological sections from 2 sentinel trout with PCR reactions showing some amplification of target DNA but slightly out of the positive-negative cutoff (CT = 38).

Table 1. Weight (Wt) and fork length (FL) data (minimum, maximum, and mean {x̄}) of fish from each exposure .

Group ID	Min. Wt. (g)	Max. Wt. (g)	x̄ Wt. (g)	Min. FL (mm)	Max FL (mm)	x̄ FL (mm)
Exposure 1	8.2	31.6	19.5	90	146	119.4
Exposure 2	10.2	33.4	21.0	93	140	121.4
Exposure 3	9.8	36.8	21.4	100	147	122.7

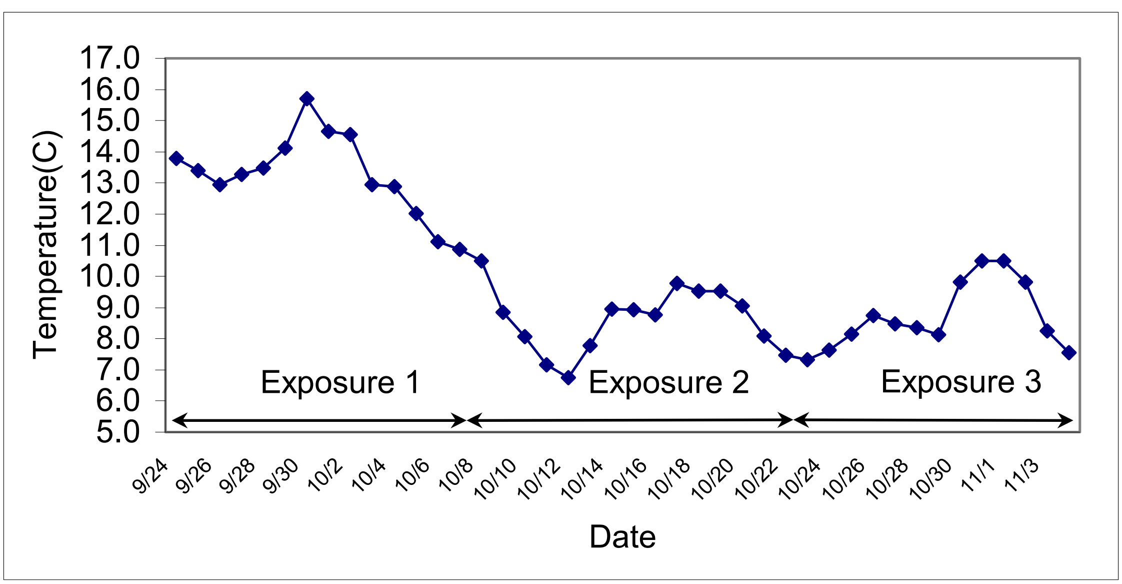
Table 2. Starting dates and number of fish exposed (in) and recovered for observation (out).

Exposure Dates	No. of Fish In	No. of Fish Out	In-River Mortalities
9/24/08	45	45	0
10/8/08	45	44	1 (jumper)
10/22/08	45	44	1

Table 3. Daily water temperature data for Bogus Creek during the 3 exposures (minimum, maximum and mean {x̄}).

Group ID	Min (ºC)	Max (°C)	x̄ (°C)
Exposure 1	8.9	17.0	13.2
Exposure 2	4.7	13.3	8.6
Exposure 3	5.3	11.5	8.6

Figure 2: Daily mean water temperature in Bogus Creek for the 3 exposures.

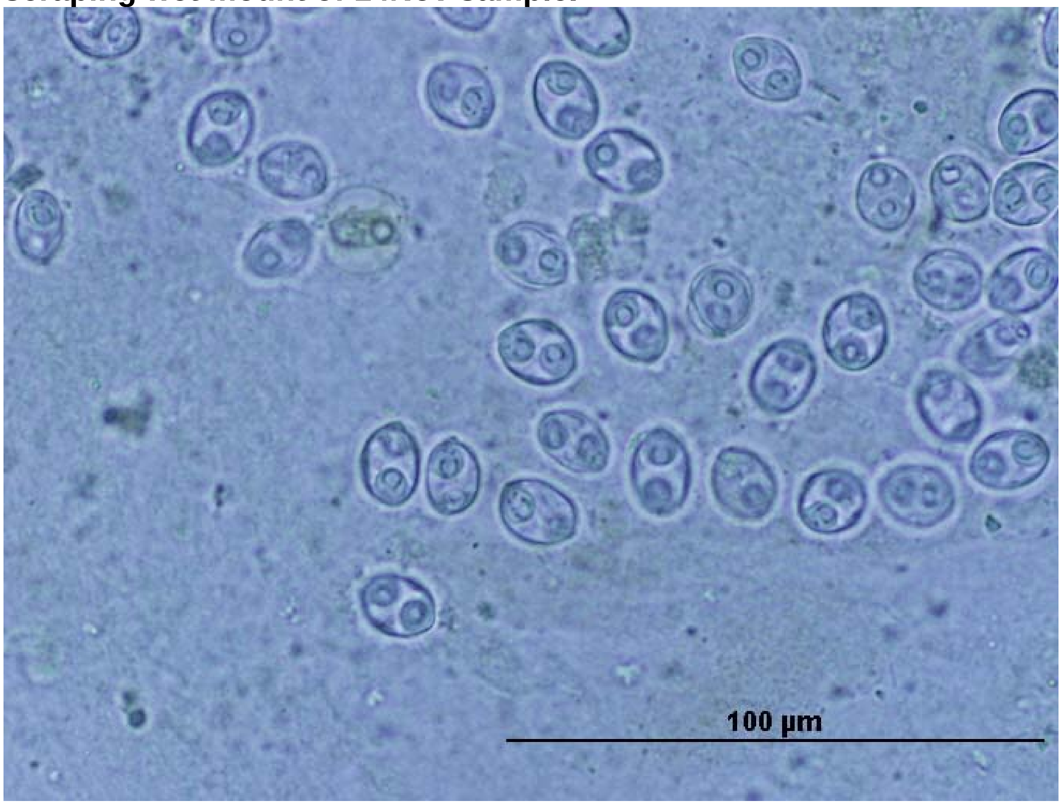


Carcass myxospore- One hundred adults were sampled in reach 2 over the 4 collection dates between 24Oct and 14Nov (Table 4). This represents approximately 6% of the 1556 adults that passed above the DFG counting weir before 10/21/08 (M. Knechtle CDFG, pers. comm.). Intestinal scrape weight represented between 8 and 15% (collection group means) of the entire intestinal weight. Cestodes and trematodes were commonly observed in the wet mounts. All females sampled had released eggs. The incidence of *C.shasta* myxospores detected in all adult intestinal tract scrapings was 30% (30 of 100 samples) over the entire collection period (Table 4). Prevalence of infection ranged from 13% in the last collection (14Nov) to 40% (both 31Oct and 05 Nov). There was wide variation in the number of myxospores / scraping within and between collection groups with no significant correlation for date of collection (Kruskal-Wallis ANOVA on ranks, H= 2.815, 3df, P=0.421), or sex (Mann-Whitney Rank sum test P=0.966). Samples from decomposed adults had significantly higher myxospore loads (median 182,109) than fresh carcasses (median 30,656)(Mann-Whitney Rank sum test P=0.036).The detection limit for wet mount examination was approximately 3,000 spores / g scraping. Large particulate matter likely reduced detection in approximately 20% of the samples. No myxospores were observed in liver, kidney, and spleen samples from 5 adults collected on 24Oct. These fish had positive intestine scraping samples (18,750 to 174,375 spores / g scraping). The large volume of this sample type (72 – 216 g) could be a factor in the lack of detection. A presumptively identified *Myxidium* sp. myxospore (Fig.3) was observed in 6 samples (2 on 31Oct, 4 on 14Nov). One scraping sample positive for *C.shasta* DNA, but negative by wet mount for *C.shasta* myxospores, contained these *Myxidium* spores (adult 71, 14Nov). It is presumed that pre-sporogonic *Cshasta* stages were present in the sample however no data exists on cross-reactivity to the *Myxidium* spore.

Table 4. *Ceratomyxa shasta* myxospore data from reach 2 Bogus Creek Chinook carcasses: Prevalence of infection and myxospores / intestinal scraping by sex and collection date.

Date	Prevalence	Mean (SD)	Range
24Oct			
Male	5 / 15 (33%)	80,258 (62,304)	18,788 – 183,094
Female	5 / 15 (33%)	59,828 (66,097)	3,206 – 171,100
Combined	10 / 30 (33%)		
31OCT			
Male	6 / 12 (50%)	537,848 (820,963)	2,825 – 2,159,500
Female	6 / 18 (33%)	3,000,808 (5.399,899)	7,125 - 13,788,600
Combined	12 / 30 (40%)		
05NOV			
Male	4 / 8 (50%)	53,092 (49,932)	3,500 – 104,300
Female	0 /2 (0%)		
Combined	4 / 10 (40%)		
14NOV			
Male	3 / 11 (27%)	5,491 (7,997,714)	7,875 – 14,668,200
Female	1 / 19 (5%)	1,400 (one fish)	
Combined	4 /30 (13%)		
Total	30 / 100 (30%)	1,287,327 (3,589,747)	

Figure 3. Presumptive *Myxidium* sp. myxospore observed in intestinal scraping wet mount of 14Nov sample.



C.shasta DNA was detected in 9 of 16 (56%) scraping subsamples from the 24Oct and 14Nov collections (Table 5). The 45% incidence of infection from scrapings, that were negative for *C.shasta* myxospores by wet mount, suggests that either a low number of myxospores were missed or DNA from pre-sporogonic stages are present in the sample.

Table 5. Prevalence of *C.shasta* DNA detection by QPCR in intestinal scrapings from 24Oct and 14Nov subsamples. Data includes the incidence of *C.shasta* DNA detection in myxospore positive (+) and undetected (UD) scrapings.

Oct 24	3 / 10 (30%)
Nov 14	6 / 6 (100%)
Incidence	9 / 16 (56%)
Myxospore +	4 / 5 (80%)
Myxospore UD	5 / 11 (45%)

Relationship to previous studies - A review of Iron Gate Hatchery (IGH) Fall-run adult *C.shasta* data, collected in 2005 – 2007, indicates that while the incidence of *C.shasta* infection is high, the myxospore stage is primarily in carcasses and not live (spawned) fish. In 2005, histological sections from spawned adults showed an 80% incidence of infection with pre-sporogonic stages (16 of 20 sections, CA-NV FHC unpublished data). In 2006, *C.shasta* myxospores were observe in only 1 of 60 intestinal scraping samples collected from spawned adults at IGH. *Cshasta* DNA was detected by QPCR in 12 of 20 (60%) of these intestinal scrapings and trophozite stages were observed in one of 20 kidney sections from the same fish (CA-NV FHC unpublished data). A similar effort, by Oregon State University and California Department of Fish and Game, to survey IGH Fall-run Chinook spawners by QPCR yielded 70% and 85% *C.shasta* DNA detection rates in 2005 and 2006, respectively (J. Bartholomew, OSU pers. comm..). In 2007, *C.shasta* myxospores were observed in the intestinal scraping of only 4% (6 of 166) of IGH spawned adults but in 34% (22 of 64) of the carcasses collected from Bogus creek, mainstem Klamath, and Shasta River (Ryan Slezak, Humboldt University, pers. comm.).

Infected juvenile salmon can also produce myxospores if they survive beyond approximately 3 weeks post-infection. In a 2008 sentinel Chinook salmon study, intestinal tracts of clinically ill fish were sampled at 20 dpe, a lower intestine content smear fixed and the remaining intestine held at ambient water temperature (18°C) for 48h. No myxospores were observed in the initial smears however the 48h samples contained 6 to 9 million myxospores / g intestine (CA-NV FHC unpublished data for 2008 Prognosis study). Bjork and Bartholomew (2008) report an average *C.shasta* myxospore load of 4.9 million from rainbow trout juveniles. Histological examination of the sentinel intestines demonstrated myxospores in necrotic muscularis layers beginning at 16 days post-exposure. The role that infected juvenile salmon play in perpetuating the lifecycle of *C.shasta* in the Klamath R. is unknown. It is unlikely that infected juvenile salmon are significant contributors of myxospores to polychaete populations in the Shasta R. to Seiad Valley “hot zone” of the Klamath River (Stocking et al. 2006) as most clinically affected juveniles are collected in reaches below this area.

We estimate an average of 4.6 billion *C.shasta* myxospores (range 1.08×10^7 - 5.28×10^{10}) were present in the Klamath River below Iron Gate dam in 2008. This estimate is drawn from the following data:

1. Approximately 12,000 adult carcasses were present in the mainstem and tributaries between Iron Gate Dam and the confluence with the Scott River in the fall of 2008.

California Department of Fish and Game video weir data (preliminary as of Jan13, 2009, M. Knechtle CDFG, pers. comm.).

Bogus video weir (as of 10/21) = 1556 plus 578 carcasses collected by Yurok Tribal Fisheries (J. Strange, 12/1/08 pers. comm..) below the weir = 2134,

Shasta video weir = 6275,
Scott R. video weir = 873,
USFWS mainstem carcass count = 2652
Subtotal = 11,934 adult Chinook

2. Incidence of myxospore infection (>3,000 spores / g scraping) = 0.30

3. 12,000 x 0.3 = 3600 myxospore positive adult carcasses

4. As *C.shasta* infection can be systemic, myxospores within the intestinal tract represents the minimum input from a given fish:
    a. Mean of 1,287,327 spores x 3600 fish = 4.6 billion (4.63×10^9 spores)
    b. Minimum detection level of 3,000 spores x 3600 = 1.08×10^7 spores
    c. Maximum observed spore level of 14.7 million x 3600 = 5.28×10^{10} spores

The fate of these myxospores within the system is unknown and an area of future research should include:

a. How many spores successfully move from the carcass into the water column and infect polychaetes in the river?
b. When do these spores leave the carcass (can carcass recovery be done at the end of the spawning season)?
c. How long do myxospores remain viable in the Klamath River?
d. How does the incidence and concentration of myxospores observed in this study compare to other Klamath R. locations (e.g. Shasta and Scott R.) or between years?

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